Metabolic characterisation of *E. coli* citrate synthase and phosphoenolpyruvate carboxylase mutants in aerobic cultures

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Abstract

E. coli is still one of the most commonly used hosts for protein production. However, when it is grown with excess glucose, acetate accumulation occurs. Elevated acetate concentrations have an inhibitory effect on growth rate and recombinant protein yield, and thus elimination of acetate formation is an important aim towards industrial production of recombinant proteins. Here we examine if over-expression of citrate synthase (*gltA*) or phosphoenolpyruvate carboxylase (*ppc*) can eliminate acetate production. Knock-out as well as over-expression mutants were constructed and characterized. Knocking out *ppc* or *gltA* decreased the maximum cell density by 14% and increased the acetate excretion by 7%, respectively decreased it by 10%. Over-expression of *ppc* or *gltA* increased the maximum cell dry weight by 91% and 23%, respectively. No acetate excretion was detected at these increased cell densities (35 g/l and 23 g/l, respectively).

Introduction

While the main goals in recombinant protein production processes with *E. coli* are high gene expression levels in high cell density cultures, those two goals can seldom be obtained simultaneously. This is caused by the excretion of considerable amounts of acetic acid under the high cell density culture conditions that are utilized in industrial processes (Contiero *et al.* 2000, De Anda *et al.* 2006). In aerobic cultures, this acetate excretion is the result of a metabolic "overflow" mechanism occurring when the carbon (glucose) flux into the central metabolic pathways exceeds the biosynthetic demands and the energy generation capacity of the cell. Generally, saturation of the tricarboxylic acid cycle (TCA cycle), and/or the electron transport chain are considered to be the main causes of this phenomenon (Contiero *et al.* 2000). Elevated acetate concentrations are highly detrimental for growth rate and recombinant protein yield (De Anda *et al.* 2006).

Since citrate synthase (CS, *gltA*) and phosphoenolpyruvate carboxylase (PPC, *ppc*) both provide a link between glycolysis and TCA cycle, they are considered to be important metabolic control points (Park *et al.* 1994, Sauer & Eikmanns 2005). Both CS and PPC direct metabolites into the TCA cycle and by diminishing the pool of PEP, pyruvate and acetyl CoA, they prevent these metabolites to participate in the acetate production (Figure 1). Moreover, over-expression of CS can increase the flux through the TCA cycle in glucose media. Many papers describe methods to reduce acetate excretion and/or its negative effects, but only a few have studied the importance of these two critical enzymes with regard to acetate production in aerobic glucose cultures. Most of the molecular approaches used to date do not completely eliminate acetate production and have a deleterious effect on growth rate or lead to undesirable accumulation of by-products (De Anda *et al.* 2006).

GltA expression is tightly regulated, since CS must fulfil the combined needs of biomass synthesis and energy production under different culture conditions. CS is an important control

point for the metabolic speed of the cell and the rate of this reaction limits the turnover of the TCA cycle under certain conditions (Walsh & Koshland, 1985). This leads to the hypothesis that controlled (over-) expression of the enzyme could decrease acetate accumulation.

PPC plays an anapleurotic role in replenishing oxaloacetate (OAA) and keeping the TCA cycle intermediates from depletion (Peng *et al.* 2004). By converting PEP to OAA, PPC prevents the accumulation of pyruvate and provides OAA, which is further converted by CS. The accumulation of pyruvate creates, according to Ponce *et al.* (1999), the highest flux to acetate and thus is the central cause of acetate production. Again, these findings lead to the assumption that over-expression of PPC can lead to less acetate formation. Lowering the pyruvate pool was also suggested by others as a means to reduce acetate production (Chao & Liao 1993, Farmer & Liao 1997, Gokarn *et al.* 2001, Lin *et al.* 2005).

Here we demonstrate that over-expression of *ppc* and *gltA* in *E. coli* MC1061 can completely eliminate acetate production and simultaneously increase the final cell density of the cultures. Knock-out and over-expression mutants were constructed and characterised at the level of expression, enzyme activity, growth and metabolite production.

Figure 1

Materials and methods

Strains and culture conditions

Escherichia coli MC1061 [(Δ*araA-leu*)7697 *ara*D139 Δ*lac*74 *gal*K16 *gal*E15 *mcr*A0 *rel*A1 *rpsL*150 *spo*T1 *mcr*B99999 *hsd*R2 λ^- F^-] was the subject of this study. *gltA* and *ppc* were PCR amplified from its genomic DNA and cloned into pGEM-T. *E. coli* DH5αF' and MA8 were used for vector propagation.

For growth experiments and enzyme activity studies, synthetic medium (SM) was used (g/l- 4 NH_4Cl, 5 NaCl, 0.5 $MgSO_4.7H_2O$, 4.8 K_2HPO_4, 3.2 KH_2PO_4, 1 leucine, 0.5 asparagine, 0.5

glutamine, 0.01 adenine, 0.01 guanine, 0.01 cytosine, 0.01 uracil, 0.01 thymine, 0.1 citrate, 0.12 Multivit Hermes (Qualiphar, Germany), 0.15 thiamine, 0.012 $CuSO_4$, 0.007 $ZnCl_2$, 0.012 $CoCl_2$, 0.008 $MnSO_4$, 0.001 $FeCl_3$, 0.003 H_3BO_3 and 0.010 $NaMoO_4$). Glucose (5 g/l) was added as carbon source. The initial pH was 6.9. Flasks were incubated at 37 °C and 200 rpm.

Genetic methods

Construction of knock-outs

The pKnock-Km suicide vector (Alexeyev 1999) was used for the construction of knock-out mutants. *ppc* was amplified using the primers '*ppc* sense' (5'-CGGTTAACGAACAATATTCCGCATTGCGTAG) and '*ppc* antisense' (5'-GCTCTAGATTAGCCGGTATTACGCATACCTG), and cloned into pGEM-T. For the construction of a knock-out mutant, pGEMT-*ppc* was cut with *Sac*II and *Stu*I, resulting in a 712 bp fragment of *ppc*. This fragment was cloned into the pKnock-Km vector. The resulting vector pKnock-Km-*ppc* was transformed into MC1061 cells.

The construction of the *gltA* knock-out was as follows: *gltA* was cloned in pGEM-T after amplification with the primers '*gltA* sense' (5'-CGAGCGCTGATACAAAAGCAAAACTCACCCTC) and '*gltA* antisense' (5'-GCTCTAGATTAACGCTTGATATCGCTTTTAAAGTCGCG). To obtain an internal *gltA* fragment, pGEMT-*gltA* was cut with *Bgl*I and *Sac*II. The 695 bp fragment was ligated in pKnock-Km and the resulting vector pKnock-Km-*gltA* was transformed into MC1061 cells.

The transformed cells were plated on LB + kanamycin and the positive colonies were tested as to the presence of plasmids and by PCR.

Construction of expression mutants

For the construction of the expression mutants, the genes were put under control of the *Trc*-promoter of the vector pOEx-HIS (Figure 2). The vector was modified by insertion of the HIS6-tag of pHisA, creating the possibility to detect the expressed genes by Western blotting. The vectors pOEx and pHisA were available at the Laboratory of Microbiology, University of Ghent, Belgium.

Figure 2

Enzyme activity assays

Preparation of cell extracts

Cells were harvested and diluted in buffer (10 mM $MgCl_2$, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7.5) to obtain an equal OD_{600}. Subsequently, the cells were broken with the Constant Systems Ltd Cell Disrupter.

Enzyme assays

The reaction mechanism for the measurement of CS and PPC activity is illustrated in Figure 3A. CS activity was measured at 37 °C as described by Morgunov & Srere (1998). The formation of CoA-SH in the reaction with CS was monitored by following the simultaneous conversion of DTNB into TNB at 412 nm. PPC activity was measured by coupling its reaction to that of CS, using an adapted reaction mixture: 100 mM Tris HCl (pH 8), 10 mM PEP, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.6 mM DTNB, 0.4 mM acetyl-SCoA. Again, production of CoA-SH was measured.

Figure 3A

Confirmation of enzyme activity loss in the knock-out mutants

The wild type and the knock-out strains were cultured in 200 ml SM to an OD_{600} of ± 2. These cultures were divided in two and harvested by centrifugation. One part was dissolved in

100 ml SM with glucose as carbon source (1%); the other part was dissolved in 100 ml SM + acetate. When the cells reached an OD_{600} of ± 2, they were harvested and lysed, and enzyme activities were measured as described above.

Confirmation of expression from the constructed expression vectors

The cells were cultured in LB + glucose (1%) to an OD_{600} of ± 2 (sample: t= -1), and then transferred to LB + IPTG (0.1 mM). A second sample of 1 ml (t= 0) was taken. Further samples were taken after 1 and 3 h growth in LB + IPTG. Enzyme expression during growth was analysed on the samples using SDS-PAGE followed by Western blotting. The enzymes were detected with anti-His6 rabbit anti-mouse immunoglobulins (alkaline phosphatase conjugate) and NBT-BCIP. In a parallel experiment, glucose (repressor) and IPTG (inducer) addition were omitted in order to study leakage expression.

After 3 h the cells were lysed, and these samples were used for enzyme activity assays.

Growth and metabolite production

The cells were cultured in SM. IPTG was added to the cultures of the over-expression mutants after 4 h. Metabolite concentrations were determined by HPLC using an Aminex HPX-87H column and a refractive index detector. The operating conditions were: mobile phase 0.5 mM H_2SO_4 at 0.6 ml/min and 65 °C.

Results and discussion

Evaluation of ppc and gltA knock-out mutants

For both genes, no plasmid DNA was found in six colonies. A PCR was done on genomic DNA of these colonies with primers specific for the viral origin of replication of the pKnock-Km. A PCR on genomic DNA of wild type cells will not result in a fragment. The results for

the wild type and for the colonies which were eventually chosen as the *ppc*-knock-out and the *gltA*-knock-out, were as expected and are shown in Figure 3B.

In order to compare the CS/PPC knock-out mutants (KO) to the wild type strain (WT), an enzyme activity experiment was performed. In this experiment the effect of acetate and glucose on the enzyme activity was also tested. The results, shown in Figure 3c, indicate that the knock-out mutants show a decrease in CS or PPC activity, which confirms the construction of the knock-out mutants. The residual activity might be due to a variety of factors, such as lack of specificity of the assay, or occurrence of revertants. As to the tests on glucose for CS, little difference is noticed between the activity of the WT and the one of the knock-out. This can be explained by the fact that glucose represses the wild type gene, and the activity of the knock-out is expected to be zero.

Glucose induces *ppc* while it represses *gltA*. Acetate induces *gltA* but has no effect on the expression of *ppc*. Nevertheless, it is an inhibitor of PPC. Neither glucose nor acetate have an effect on the activity of the enzyme CS (Morikawa *et al.* 1980, Park *et al.* 1994). In Figure 3C a much higher activity of the wild type CS can be observed on acetate compared to glucose, which confirms the hypothesis of induction and repression, respectively. The inhibition of PPC by acetate and the induction of it by glucose can be seen as a slight decrease in activity on acetate compared to glucose.

Figure 3B and C

Evaluation of ppc and gltA expression mutants

For the construction of the expression mutants, the genes were cloned in the vector pOEx-His (Figure 2). The resulting vectors pOEx-His-*ppc* and pOEX-His-*gltA* were transformed into competent MC1061 cells.

Transformation of *E. coli* MC1061 with pOEx-His-*ppc* and pOEX-His-*gltA* yielded 10 colonies and 4 colonies, respectively. The plasmids from these colonies were checked by

restriction analysis: 3 colonies from *E. coli* pOEx-His-*ppc* displayed the expected restriction pattern, while 2 of *E. coli* pOEx-His-*gltA* gave the expected results (data not shown). One colony of each was chosen as the over-expression mutant (OE) of PPC and CS, respectively.

To confirm that PPC and CS were expressed from the new expression vectors in the OE mutants, an induction experiment was set up. The results are shown in Figure 4. The experiments show a significant production of CS and PPC upon IPTG induction in the OE mutants (Figure 4A), clearly increasing in time. However, leakage expression was also detected in the presence of glucose (t= -1) before IPTG addition. This leakage expression was confirmed in the parallel test without IPTG induction; the leakage expression being much more noticeable for CS than for PPC. As expected, no expression can be detected by Western analysis in the wild type. With this test, the expression of PPC and CS from the vectors pOEx-His-*ppc* and pOEX-His-*gltA* was confirmed.

The activities of PPC and CS, which were measured in the samples taken after 3 h of induction, are presented in Figure 4C. IPTG induction as well as leakage expression of PPC and CS cause a considerable increase in CS or PPC activity in the over-expression mutants. Taking into consideration the results of the Western analysis and these activity measurements, one can decide that even a little increase in enzyme production has a major effect on enzyme activity. The lower activity of PPC with induction than without it could be explained by the formation of inclusion bodies. The activity increase for the wild type after IPTG addition could be attributed to the overall effect of this compound on the metabolism. These tests clearly illustrate that PPC and CS are over-expressed and that the produced enzymes are active.

Figure 4

Growth and metabolite production

To study the effect of PPC or CS over-expression or elimination on the growth and metabolite production of *E. coli*, growth experiments were carried out in which the wild type strain, the knock-outs, the over-expression mutants and the complemented knock-outs were compared. The complemented knock-outs were obtained by transformation of the knock-out strains with pOEx-His-*ppc* or pOEX-His-*gltA*. The results of the growth experiments are depicted in Table 1. The cell dry weight (CDW), the pH, the consumption of glucose and the formation of acetate, citrate, formate and malate were monitored. No other peaks were observed in the HPLC outputs.

The *ppc* knock-out reached a lower cell density than the wild type and grew more slowly, which confirms the results of Peng *et al.* (2004). The citrate production of this knock-out is the highest of all strains tested and it excretes 1.5 g malate/l. The acetate excretion has increased a little, despite the lower cell density. This contradicts results of Peng *et al.* (2004) who observed less acetate excretion in the mutant. A higher acetate excretion can be justified easier, because knocking out the PPC enzyme leads to an increase in the PEP pool, and consequently via an increase in pyruvate and acetyl-CoA to more acetate (Ponce 1999). These results indicate that knocking out *ppc* has a negative effect on the cell metabolism: growth is impaired and the excretion of metabolites increases.

The *gltA* knock-out also reached a lower CDW than the wild type, but at the same time excretes 10% less acetate and the final pH of the culture is higher than that of the wild type. No explanation could be found for the high citrate concentration in the *gltA* knock-out cultures.

Over-expression of PPC clearly has a positive effect on growth to higher cell densities, which is accompanied by the elimination of acetate formation and thus, with a better pH-profile of the cultures. The CDW is almost twice as high in cultures of the over-expression mutant as in

cultures of the wild type or the knock-out strain. This confirms that a higher PPC activity is associated with a higher flow through the TCA cycle (no more acetate excretion), a higher NADH availability and more ATP, resulting in a higher biomass production. The expression mutant had a longer lag-phase than the wild type strain, but afterwards grew with approximately the same growth rate, while consuming far less glucose. This confirms and even tops the results obtained in previous studies: Chao & Liao (1993) and Holms (1996) obtained a 60% decrease in acetate production without affecting growth rate, while here a complete elimination of acetate excretion is found. The formate production is not much affected, but the formation of citrate has tripled. Contradictory, this higher citrate excretion was also observed in the knock-out of PPC.

The positive effect of the over-expression of PPC on growth to higher cell densities and acetate production is not as obvious when over-expressing CS. Nevertheless, it is still noteworthy. The cell density increased with 23% after over-expression of CS, while the pH did drop lower than in the wild type cultures. No acetate was detected for the over-expression mutant. The complemented *gltA* knock-out excreted a maximum amount of acetate of 2.4 g/l, but it could be observed that this strain consumed the acetate during further growth. In this way the strain was able to grow to a cell density as high as the one observed with over-expression mutant. The growth rate of this strain was comparable to that of the wild type. Over-expression of *gltA* had a variable influence on the formate production and again tripled the citrate formation. Nor malate, nor lactate was detected in the cultures of the CS mutants.

Table 1

Conclusion

In this study we have shown that an increased expression of *ppc* or *gltA* makes it possible to completely eliminate acetate excretion. Over-expression of phosphoenolpyruvate carboxylase doubled the maximum cell density of the *E. coli* cultures, and over-expression of citrate

synthase increased it with 23%. The growth rates of the over-expression strains were comparable to the wild type. Leakage expression experiments revealed less PPC activity with than without IPTG, a finding which may be important for cost reduction in future industrial applications.

It can be concluded that over-expression of *ppc* and *gltA* can lead to a higher flux through the TCA cycle and thus it can eliminate the acetate production by eliminating saturation of this cycle. In further experiments, the effect of *ppc* and *gltA* expression, and the resulting elimination of acetate excretion, on the production of (heterologous) proteins will be investigated. The elimination of acetate production and its accompanying carbon waste is a valuable feature that should facilitate the attainment of very high cell concentrations and productivities (De Anda *et al.* 2006). Koo & Park (1999) observed a 60% increase of recombinant protein production in *E. coli* after elimination of the acetate production. It is tempting to speculate that similar promising or even better results will be achieved with our new expression mutants.

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Table 1. Maximal cell dry weight (CDW), minimal pH and maximal acetate, formate, citrate and malate concentration during growth of MC1061 and *gltA*/*ppc* mutants (KO *gltA*$^{-}$/*ppc*$^{-}$, *gltA/ppc* knock-out; KO *gltA*$^{+}$/*ppc*$^{+}$, complemented *gltA/ppc* knock-out; MC1061 *gltA*$^{+}$/*ppc*$^{+}$, *gltA/ppc* over-expression mutant). Lactate production was observed in none of the cultures. Concentrations are given in g/l (N.D. = none detected).

Table 1 Growth characteristics of MC1061 and *gltA*/*ppc* mutants

	MC1061	KO *gltA*$^-$	KO *gltA*$^+$	MC1061 *gltA*$^+$	KO *ppc*$^-$	KO *ppc*$^+$	MC1061 *ppc*$^+$
CDW	18.4	15.8	**22.1**	**22.7**	15.8	**26.4**	**35.1**
pH	5.0	5.4	5.0	4.7	5.0	**6.0**	**6.0**
acetate	3.0	2.7	2.4	**N.D**.	3.2	**N.D.**	**N.D.**
formate	0.6	0.7	0.4	1.1	0.4	1	1
citrate	1.3	3	3.5	3.3	2.8	4.3	3
malate	N.D.	N.D.	N.D.	N.D.	1.5	N.D.	N.D.
lactate	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Figure 1. The TCA cycle of *E. coli*. Over-expressed enzymes are indicated with bold arrows.

Figure 2. Construction of pOExHis, pOEx-His-*ppc* and pOEx-His-*gltA*. A 2717bp fragment of pOEx containing a part of the tetracycline resistance gene (*TetR*), the *lacIq* allele and the strong *trp/lac* promoter was ligated to a fragment of pHisA containing a His6-tag, a multi-cloning site, two transcription terminators (rrnBT1T2 and TrpA), a replication ori and the rest of the *TetR*. This ligation resulted in the expression vector pOEx-His. One sample of pOEx-His was cut with *Xma*III and *Xba*I (sticky), which resulted in a fragment containing a part of the *TetR* and TrpA and rrnBT1T2. Another sample was cut with *Xma*III and *Bbr*PI (blunt after His6-tag), creating a fragment which contains the rest of the *TetR* and the *Trc*-promoter. The genes *ppc* and *gltA* were obtained by cutting pGEMT-*ppc* and pGEMT-*gltA* with *Hpa*I (blunt, 5') and *Xba*I (sticky, 3'), and *Eco*47III (blunt, 5') and *Xba*I (3'), respectively The obtained fragments and the genes *ppc* and *gltA* were ligated, resulting in pOEx-His-*ppc* and pOEx-His-*gltA*.

Figure 3. A. CS and PPC reaction monitored by TNB (thionitrobenzoate) formation from DTNB (dithionitrobenzoate).

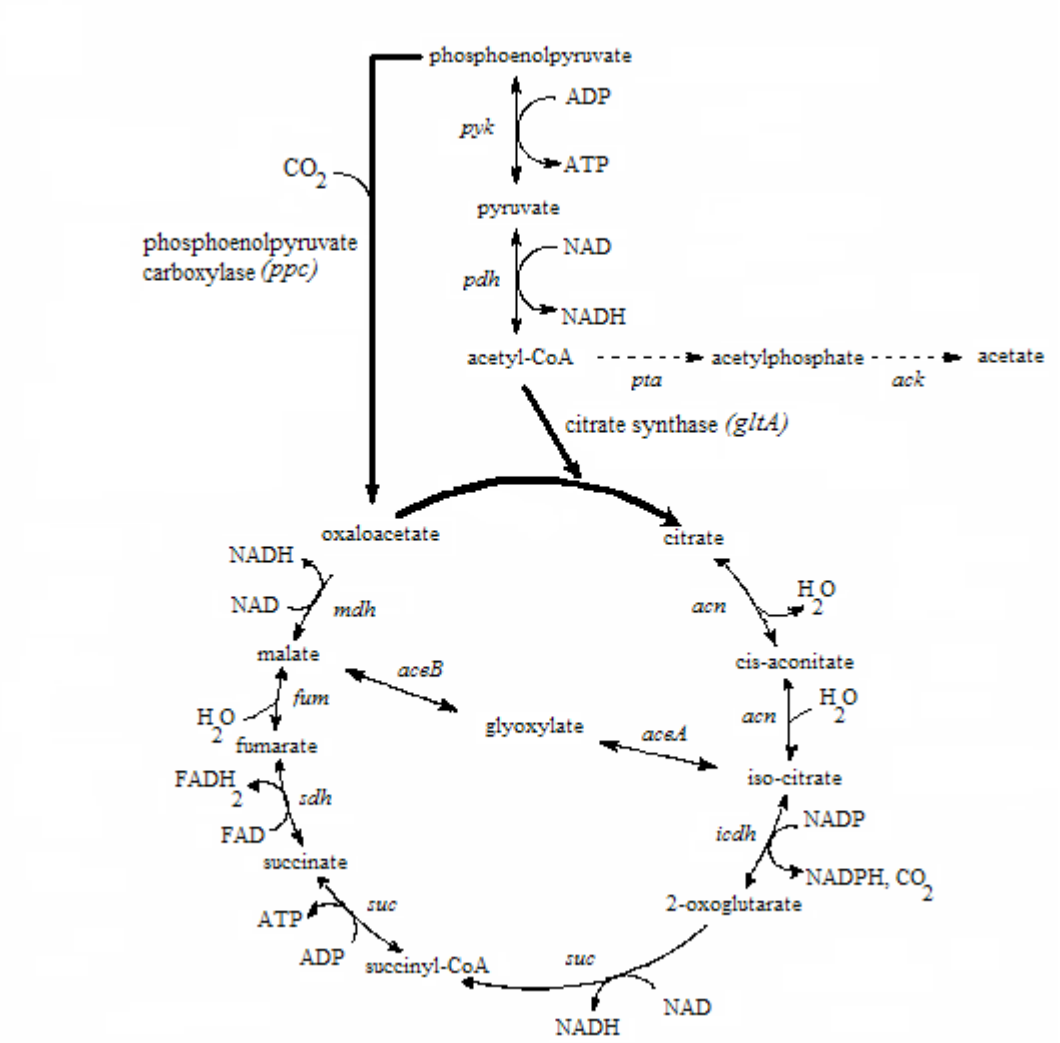
B. Verification of the integration of the pKnock-Km suicide vector in the genome of the *ppc*- and *gltA* knock-out: a fragment of 725bp is expected when the vector is present in the genome. Lane 1: marker (Smart Ladder, Eurogentec). Lane 2: wild type. Lane 3: *gltA* mutant. Lane 4: *ppc* mutant. Lane 7: blanc. Lane 8: marker.

C. Relative enzyme activities of CS and PPC in *E. coli* MC1061 (WT) and knock-out mutants (KO)

Figure 4. A - B. Western blot detection of PPC and CS after IPTG induction (A) or during leakage expression (B; A: t=-1). Lane M: Marker (BenchMark Prestained Protein Ladder, Invitrogen)

C. Relative enzyme activity for expression mutants compared to that of the wild type strain

Figure 1.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CO2
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pdh
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pta
acetylphosphate
ack
acetate
citrate synthase (gltA)
oxaloacetate
citrate
NADH
NAD
mdh
acn
H2O
malate
cis-aconitate
aceB
fum
H2O
glyoxylate
aceA
acn
H2O
fumarate
iso-citrate
FADH2
sdh
FAD
icdh
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ADP
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NAD
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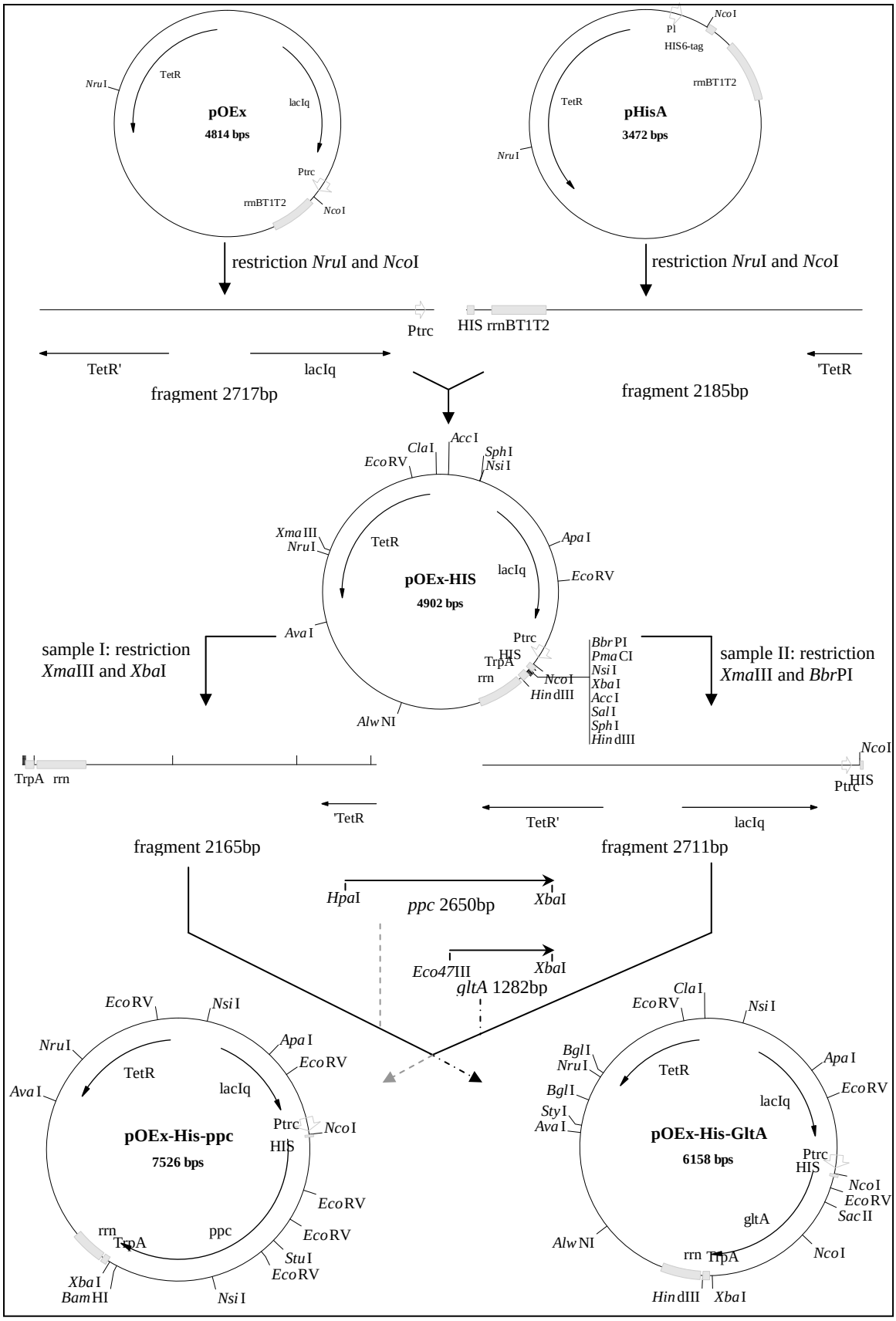
Figure 2.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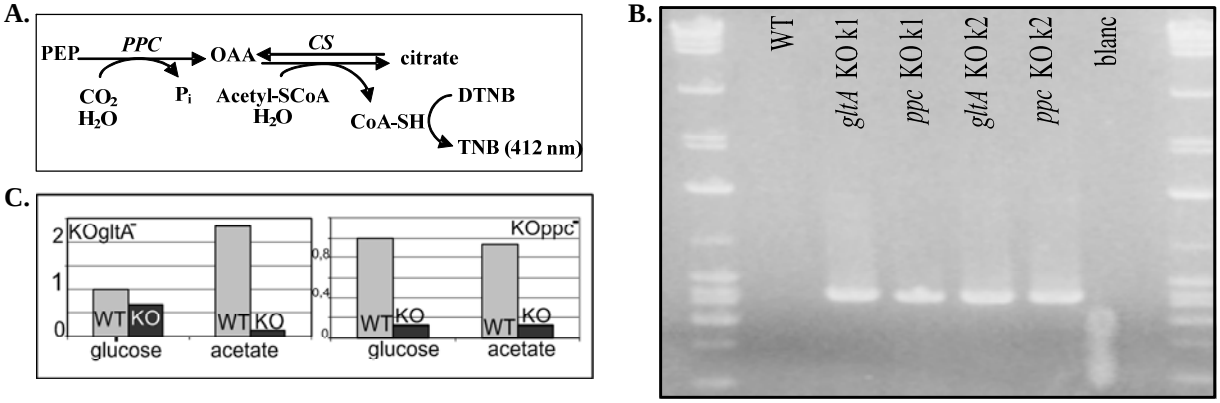
HindIII
XbaI

Figure 3.


A.
PEP
PPC
OAA
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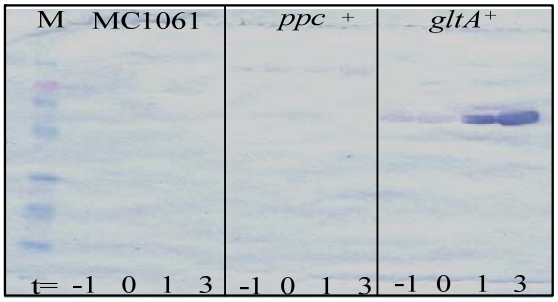
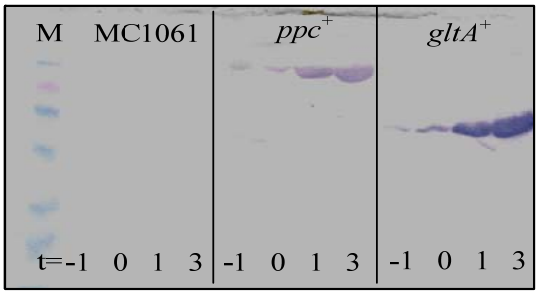
Pi
Acetyl-SCoA
H2O
CoA-SH
DTNB
TNB (412 nm)
B.
WT
gltA KO k1
ppc KO k1
gltA KO k2
ppc KO k2
blanc
C.
KOgltA
KOppc
WT
KO
glucose
acetate

Figure 4

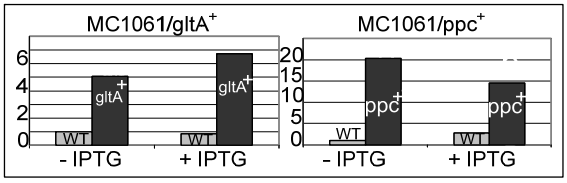
A.


M
MC1061
ppc +
gltA+
t= -1 0 1 3
-1 0 1 3
-1 0 1 3

B.


M
MC1061
ppc+
gltA+
t=-1 0 1 3
-1 0 1 3
-1 0 1 3

C.


MC1061/gltA+
MC1061/ppc+
6
4
2
0
20
15
10
5
0
gltA+
gltA+
ppc+
ppc+
WT
WT
WT
WT
- IPTG
+ IPTG
- IPTG
+ IPTG